Chapter 4

Synergistic Effects of Pretreatment Process on Enzymatic Digestion of Rice Straw for Efficient Ethanol Fermentation

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Additional information is available at the end of the chapter

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1. Introduction

Lignocellulose, the most abundant renewable biomass produced by plants from photosynthesis, has a yearly supply of approximately 200 billion metric tons worldwide (Ragauskas et al., 2006; Zhang et al., 2006). Lignocellulosic biomass is widely expected to be a major resource for biorefineries, including bioethanol (Lin and Tanaka, 2006). The composition of lignocellulose varies depending on plant species, plant parts, growth conditions, etc. (Ding and Himmel, 2006; Zhang and Lynd, 2004), and their structures are rigid and low degradable against cellulase enzymes. In general, the lignocellulose structure is composed by three major components: crystalline cellulose, amorphous hemicellulose and non-sugar lignin. Cellulose microfibrils are coated with hemicellulose matrices building holocellulose structures and severely protected by lignin outside. The structures are rigidly packed to form a physical barrier for cellulase access to cellulose chains (Mansfield et al., 1999). To hydrolyze them efficiently into sugars, a high dosage of commercial available cellulase enzymes is required. At a current technical stage, 20 g-cellulase is needed to hydrolysis 1 kg cellulose at 70% for 5 days (Gusakov, 2011; Roche et al., 2009). However, the baseline production cost of cellulase is still expensive as reported to be $10.14/kg (Klein-Marcuschamer et al., 2012). If the recalcitrance problem remains unresolved, it is not feasible for high-solids enzymatic saccharification.

The ability of cellulase access to cellulose chains within microfibrils will be limited even if lignin is completely removed from the cellulose structure, because its ability is generally limited to accessing the outer layer of the microfibrils (Mansfield et al., 1999). Although cellulose can be slowly eroded by surface shaving or planning, cellulose chains in highly ordered and tightly packed regions of microfibrils must be disintegrated by delamination,

disruption or loosening to increase the surface area and make individual molecule more accessible and available for interaction with cellulase (Ishizawa et al., 2009; Ragauskas et al., 2006). For this reason, the pretreatment must be implemented before enzymatic saccharification and also required to facilitate amorphogenesis as the initial stage in the enzymatic hydrolysis of cellulose (Coughlan, 1985; Din et al., 1991; Teeri et al., 1992).

Among various methods available for biomass pretreatment, chemical delignification and swelling were investigated in this study, because these processes could show the similar effect to amorphogenesis of cellulose fiber by cellulase. Most of the chemical processes presently used, however, might not be preferable to saccharification, because of incomplete lignin removal and degradation of polysaccharides and loss of hemicelluloses (Fang et al., 1999; Sun et al., 2004). Most processes require high chemical charges to attain the complete lignin removal, because the single pretreatment process is not effective when performed at low chemical charges. In some cases, rearrangement of the lignin structure occurs during the pretreatment process (Kumar and Wyman, 2009). Products released by the degradation of polysaccharides and hemicellulose extracts strongly inhibit the cellulose hydrolysis by cellulase (Jing et al., 2009).

The pretreatment process should be designed to remove lignin and to disintegrate the cellulose structure without loss (degradation) of cellulose and hemicellulose parts (Frey-Wyssling, 1954; Peterlin and Ingram, 1970; Morehead, 1950). Pretreatment with sodium chlorite acidified by acetic acid (acidified sodium chlorite) perhaps meets the requirement of delignification and effectively solubilizes lignin at moderate temperatures. It is noteworthy that the acidified sodium chlorite delignification causes only trace solubilization of glucan and xylan (Ahlgren and Goring, 1971). It is also reported that sodium bicarbonate is effective to disintegrate the cellulose structure and the swelling by carboxylation of produced fiber (Kwasniakova et al., 1996).

In this study, the advanced pretreatment process for enzymatic hydrolysis of rice straw has been demonstrated by combining the delignification by acidified sodium chlorite with the disintegration of cellulose structure and the alteration of crystalline structure by swelling with sodium bicarbonate. The efficiency of the pretreatment process on saccharification and fermentation (based on simultaneous saccharification and fermentation process) of rice straw was evaluated by using commercially available cellulase.

2. Materials and methods

2.1. Materials and microorganism

Sun-dried rice straw of Koshi-hikari (Niigata-ken, Japan) was used as a source of lignocellulosic biomass. Chemical composition of rice straw was generally ranged from 24% to 38% cellulose, 12% to 22% hemicellulose and 16% to 20% lignin, based on the dried weight. Microcrystalline cellulose (<20 μm particle size) was purchased from Merk (Darmstadt, Germany). Avicel PH-101 (<50 μm particle size) was purchased from Sigma-Aldrich (St. Louis,

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MO). The digestive enzyme mixtures of Novozym 188 (372 β-glucosidase IU/g, source of β-glucosidase) and Celluclast 1.5L (64 FPU/g, 16 β-glucosidase IU/g, source of endo-/exo-type cellulase) obtained from Novozymes A/S (Bagsværd, Denmark) were used for enzymatic saccharification. Milli-Q grade water (18.2 MΩcm resistivity) was used throughout all the experiments. For fermentation, wild-type yeast strain *Saccharomyces cerevisiae* NBRC2114, obtained from National Bioresources Research Center (NBRC, Ibaraki, Japan), was used. This strain can produce ethanol anaerobically from glucose but not from pentose sugars such as xylose, arabinose and ribose.

2.2. Sample pretreatment

The pretreatment of rice straw with acidified sodium chlorite was performed in a water bath using sodium chlorite and acetic acid at 80 ºC according to a modified literature method (Hubble and Ragauskas, 2010). Rice straw samples were ground using a laboratory cutting mill to a particle size on the order of 5 mm, impregnated by immersion in a flask containing deionized water (60 ml/g solid) at 25 ºC for 3 days to form solids slurry. The delignification was started by addition of glacial acetic acid (0.04 ml/g solid) and sodium chlorite (0.4 g/g solid) to solids slurry. The mix was heated to 80 ºC with gentle swirling at intervals. Fresh amounts of acetic acid and sodium chlorite were added until the samples were judged to be sufficiently delignified by the persistence of yellowish-green chlorine dioxide gas that was generated on mixing the reagents (normally after 1 h for one reaction).

For swelling of delignified rice straw, the samples were initially impregnated by immersion in a flask containing sodium bicarbonate solution at 0.5% (wt./vol.) at 25 ºC for 24 h. After autoclaving at 122 ºC for 20 min, the samples were washed until the solution was colorless and neutral in pH. All samples were sun-dried for at least 3 days and stored in desiccators at 25 ºC until used.

2.3. Scanning electron microscopy

The electron microscopic study of pretreated rice straw was performed with FE-SEM (Hitachi S-4700 Type II; Hitachi, Tokyo) after the dried samples were placed on a conductive carbon tape and coated with Pt-Pd using a sputter coater (Hitachi E102 Ion Sputter; Hitachi) for 2 min at DC±20 mA as previously mentioned (Kahar et al., 2010).

2.4. XRD and FTIR analysis

X-ray diffraction (XRD) analysis on avicel, cellulose microcrystalline, untreated and pretreated rice straw were conducted according to a method described by Chang and Holtzapple (2000). Samples of particle size less than 125 mm were scanned on a RIGAKU-D/MAX instrument (Uitima III, Japan) at a speed of 1º/min, range from 2θ = 0º-40º, and with a step size of 0.04º at 25 ºC. Crystallinity index (CrI) was calculated according to the method described by Segal et al. (1959).

For FTIR analysis, the ground samples were prepared by pressing 2 mg of cellulosic samples on 200 mg of spectroscopic grade potassium bromide (KBr). The spectra were recorded in the

middle IR range 3500-750 cm^{-1} using a JASCO FT/IR4200 Spectrometer with detector at 4 cm^{-1} resolution and 40 scans per measurement. Essential FTIR (Operant LLC, Sydney, Australia) software was used as a tool for analysis of IR spectra.

2.5. Enzymatic saccharification

A batch enzymatic hydrolysis was conducted at 1% (wt./wt.) of dry solid loading in a 0.1 M acetate buffer (pH 4.8) containing 0.02% (wt./vol.) sodium azide. The total working volume was 100 in a 300 ml flask. Before the addition of cellulase enzymes, the mixture of substrate and buffer was preheated in an incubator shaker at 50 °C for 30 min to allow the substrate to disperse uniformly in the buffer. Celluclast 1.5L and Novozym 188 were added into tubes immediately to initiate enzymatic hydrolysis. The saccharification was occurred under the temperature of 50 °C for 24 h. To finish the reaction, the mixtures were immediately placed over a boiling water bath for 5 min to deactivate the enzymes as described by Helle et al. (1993) and Desai and Converse (1997). After enzyme inactivation, each sample was centrifuged for 5 min at 8,000 × g, and supernatants were collected. The supernatant samples were stored at 4 °C for subsequent sugar analysis.

2.6. Fermentation

Fermentation was performed anaerobically in 2-l jar fermentor, equipped with pH and dissolved oxygen concentration monitoring system (FermExpert, BEM, Ibaraki). Prior to fermentation, yeast culture was prepared by inoculating a single colony of NBRC2114 strain in YM medium, which containing bacto peptone (0.5%, wt./vol.), bacto yeast extract (0.3%, wt./vol.), bacto malt extract (0.3%, wt./vol.), glucose (1%, wt./vol.), xylose (1%, wt./vol.) and aerobically cultured at 30 °C overnight. For fermentation, minimal medium (MM) containing bacto yeast nitrogen (without amino acids and ammonium sulfate) (0.17%, wt./vol.) and ammonium sulfate (0.5%, wt./vol.) supplemented with pretreated rice straw was used. After transferring the yeast culture into MM, the fermentation started by the addition of cellulase enzyme mixture at a final loading of 10, 20, 100, 200 (g-biomass/g-enzymes). The solution of 5 N NaOH was used to keep the pH of culture at around 5. To maintain the anaerobic condition at the initial stage of fermentation, a continuous stream of sterile nitrogen gas (0.1 VVM [volume of air/volume of reactor × minutes]) was flowed through the sterilized membrane filter into the reactor. The gassing was stopped upon cell production of sufficient gases (positive headspace pressure), usually between 12 and 24 h

2.7. Analytical methods

The Klason lignin content of the samples was determined using the Laboratory Analytical Procedures (LAPs) provided by the National Renewable Energy Laboratory (NREL) (Sluiter et al., 2008). The amount of total sugars was determined as reducing sugars by 3,5-dinitrosalicylic acid (DNS) assay, as described by Miller (1959).

To determine the concentration of byproduct ions upon acidified sodium chlorite treatment, ion chromatography analyses were carried out with a Dionex ICS -1500 High

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Performance Integrated Ion Chromatography System equipped with an Auto suppressor system. The columns of ION PAC AS23 and ION PAC AG23 were used as a main isolation column and a guard column, respectively, for the determination chloride, chlorite and chlorate ions were used at a flow rate of 1 mL/min, with the elution program consisting of an isocratic elution with 4 mM $NaHCO_3$/0.4 mM Na_2CO_3 buffer at 30 °C. The spectrophotometric analysis at 359 nm was used to determine the concentration of chlorine dioxide.

The protein concentration was measured by the Bradford protein assay using bovine serum albumin (BSA) as a standard (Bradford, 1976). All the experimental results were the average of triplicates, unless specified otherwise.

3. Results and discussion

3.1. Delignification of rice straw by pretreatments with acidified sodium chlorite and sodium bicarbonate

Since lignin is arranged in multiple lamellar sheets of lignocellulose matrices, a single batch reaction of chemical pretreatment is occasionally not sufficient to achieve a complete delignification (Wise et al., 1946; Klein and Snodgrass, 1993). In this study, the delignification of rice straw by acidified sodium chlorite was evaluated by repeating one hour batch reaction from one time to four times (1x to 4x). Figure 1A shows the residual lignin content of rice straw after pretreated and the control. The lignin content of rice straw decreased to about 38% (wt./wt.) of the control by one time treating (1x) and then decreased to 16% (wt./wt.) after four times repetition (4x). Interestingly, much higher rates of lignin removal were achieved when the sodium bicarbonate treatment was additionally applied after the chlorite treatment. For example, in case of three times repetition (3x) of the chlorite treatment, the additional processing with sodium bicarbonate (3x+swelling) resulted in more decreasing of lignin content at 8% (wt./wt.) against 20% (wt./wt.) in the original 3x.

A previous study (Hubbell and Ragauskas, 2010) reports that the acidified sodium chlorite treatment should be sufficient to remove lignin from cellulose samples with lignin content below 30% (wt./wt.) when the reaction was repeated two times, and it should be performed at least three times for higher lignin contents. However, the work was conducted on the pure cellulose matrix artificially coated with lignin at appropriate concentrations. This means that the reported process could be implemented for removal of surface lignin, but it was not clear whether the process was effective for removal of integrated lignin including internal lignin. According to our study, three times repeated delignification (3x) was not enough, resulting in only about 80% (wt./wt.) removal as shown in Fig. 1(A), unless the swelling by sodium bicarbonate was applied. Therefore, the swelling seems to play an important role, not only in removal of surface lignin, but also in removal of integrated lignin.

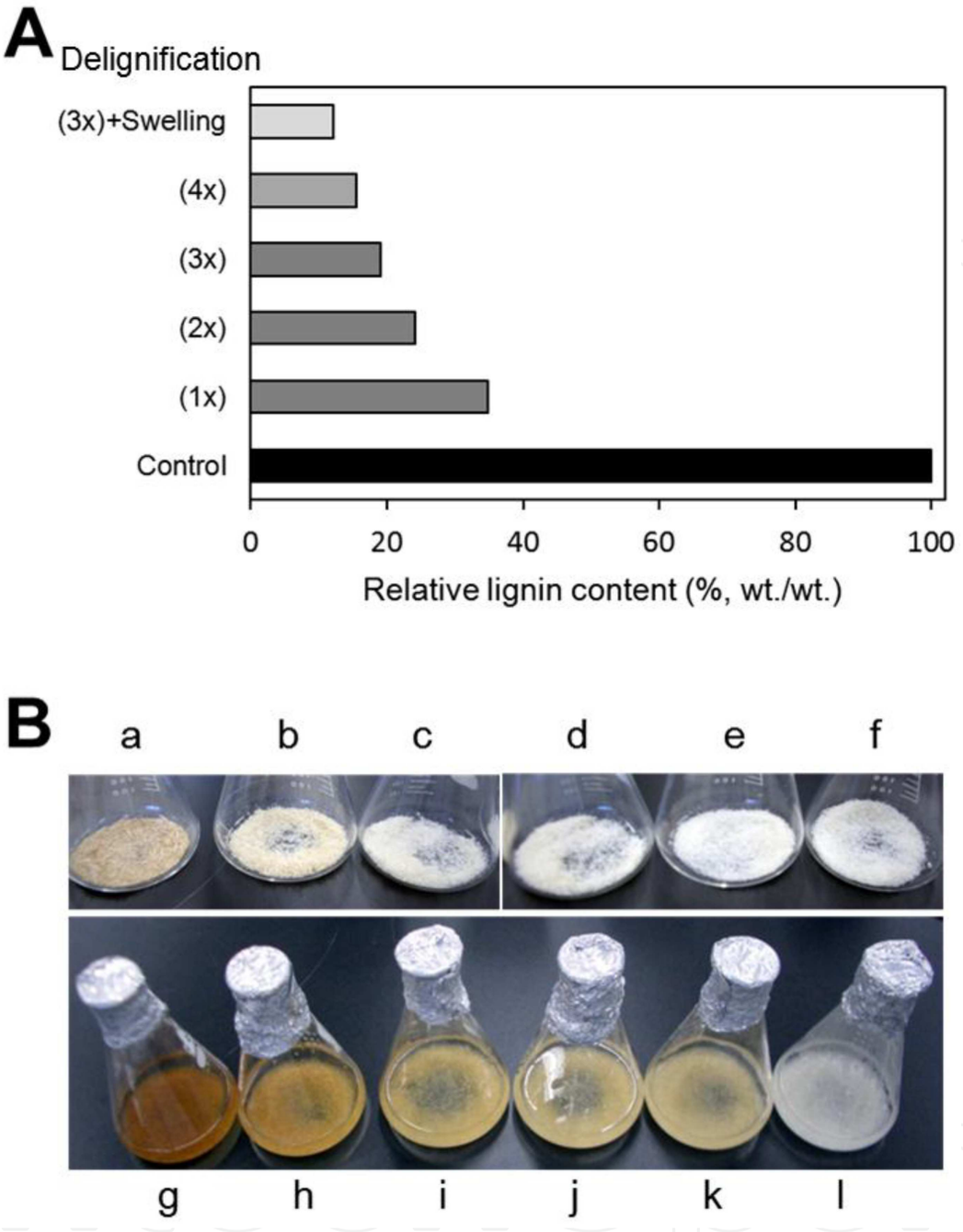

Figure 1. Delignification of rice straw. A) Effects of delignification by acidified sodium chlorite (treated 1x to 4x) and swelling by sodium bicarbonate on the lignin contents of rice straw. B) Photographs of dried and autoclaved rice straw fibers after treated with acidified sodium chlorite (1x)(b, h), (2x) (c, i), (3x) (d, j), (4x) (e, k), acidified sodium chlorite (3x) and sodium bicarbonate (f, l). As a control, untreated rice straw was used (a, g). Heat-treatment by autoclaving was performed at 122 ºC for 20 min, just after impregnation of the samples in water.

To confirm the presence of internal lignin in the rice straw, the samples were immersed in water (2.5 g-solid/150 ml) and then autoclaved at 122 °C for 20 min. Lignocellulosic materials were tanned after processed with hot water due to the denaturation of lignin components to hot-water-extractable tannins (Allen et al., 1974). As shown in Fig. 1(B), the

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rice straw sample treated with sodium bicarbonate after chlorite were not tanned (l), in contrast to the control (g) and the sample treated with chlorite only for 2x (i) to 4x (k), even though they were bleached as colorless solids prior to hot-water processing (a, c, d, e, f). This result indicates that the internal lignin could be efficiently removed by swelling, not by acidified sodium chlorite treatment.

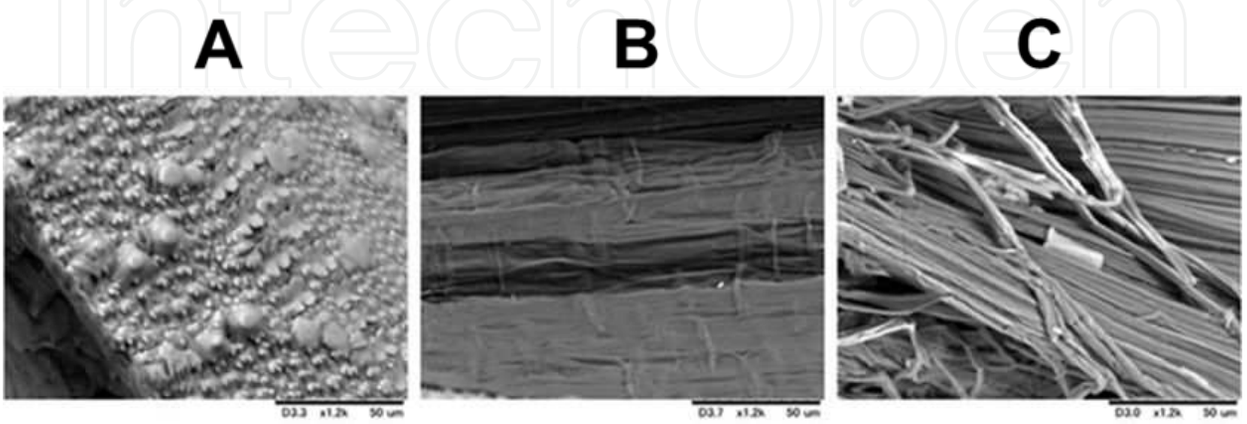

Figure 2. SEM images of untreated and pretreated rice straw. (A) Untreated rice straw. (B) Rice straw after treated with acidified sodium chlorite (3x). (C) Rice straw after treated with acidified sodium chlorite (3x) and sodium bicarbonate.

The delignification of rice straw was further visually evaluated by SEM analysis (Fig. 2). Observed by SEM were rice straws treated with acidified sodium chlorite three times (3x) (B), treated with acidified sodium chlorite plus sodium bicarbonate (3x+swelling) (C) and treated only by autoclaving (122 °C, 20 min) as control (A). As clearly shown in Fig.2(C), the integrated lignin was almost completely removed from cellulose structures by the 3x+swelling treatment so that cellulose fibrils appear to be separated and accessible to the enzymes. The 3x treatment partially removed lignin from the surface, but cellulose fibril bundles still remained, supposedly leaving internal lignin.

The efficiency of lignin removal on treated rice straw was evaluated in detail by XRD (Fig. 3) and FTIR analysis (Fig. 4). XRD analysis was performed using pure cellulose (microcrystalline cellulose) as control (Fig. 3A). The observed crystallinity index (CrI_{obs}) of the samples were 48%, 53%, 67% for autoclaved (without chemicals), acidified sodium chlorite (3x) treated, acidified sodium chlorite (3x) and sodium bicarbonate treated samples, respectively, while the CrI_{obs} of pure cellulose was 68%. Treatment with sodium bicarbonate after acidified sodium chlorite (3x) increased the CrI_{obs} of the samples to closer with that of pure cellulose. This result indicates that more non-cellulosic components (lignin included) removed from the cellulose structures. The increasing of CrI_{obs} of rice straw after treated with acidified sodium chlorite (3x) was not significant unlike those after treated with acidified sodium chlorite (3x) and sodium bicarbonate. It means that surface lignin was removed by acidified sodium chlorite (3x), but internal lignin could be removed only by treatment with sodium bicarbonate.

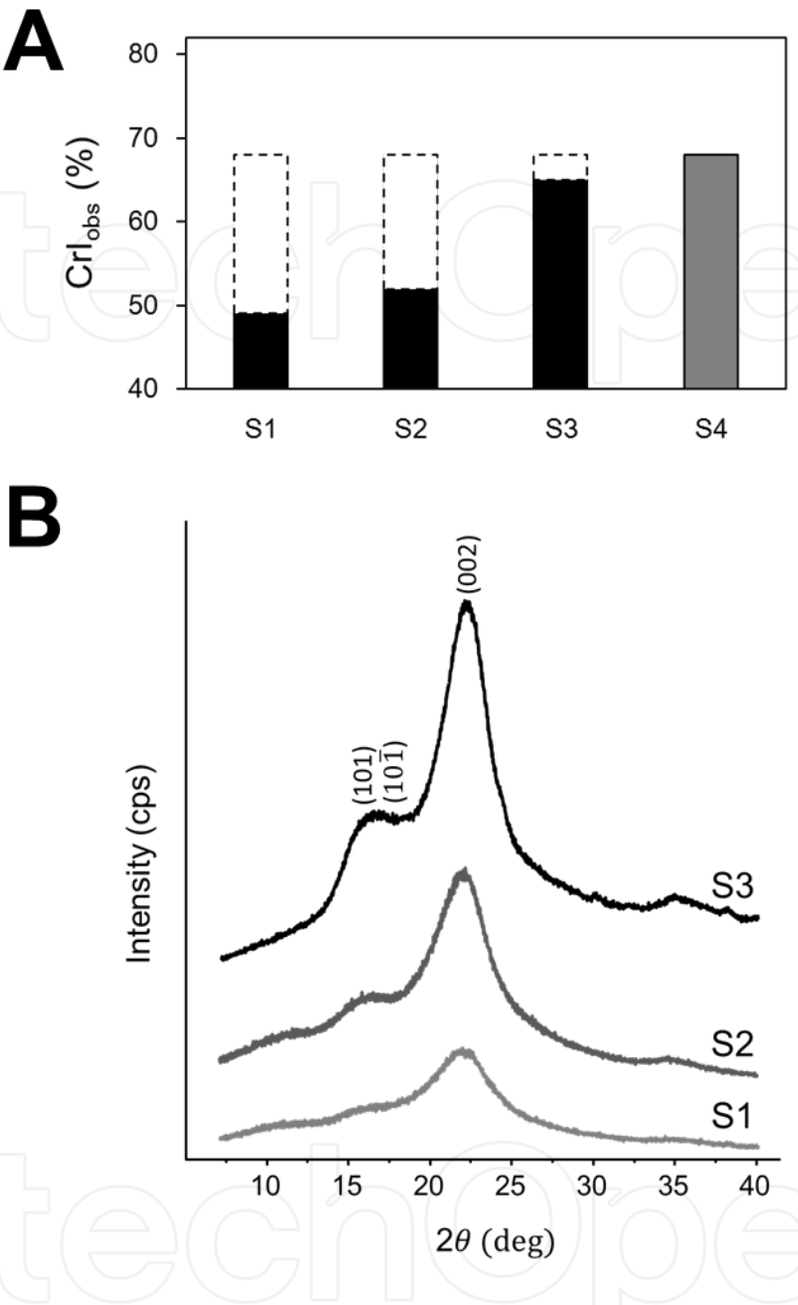

Figure 3. XRD analysis of pretreated rice straw. (A) The levels of crystalline index (CrI_{obs}) of rice straw compared to pure cellulose solids. (B) XRD spectra of rice straw samples. (S1) Autoclaved rice straw. (S2) Rice straw after treated with acidified sodium chlorite (three times). (S3) Rice straw after treated with acidified sodium chlorite (3x) and sodium bicarbonate. (S4) Microcrystalline cellulose (20 μm).

The XRD spectra of treated rice straws are shown in Fig. 3B. The intensities of peaks corresponding to (101), (10$\underline{1}$) and (002) lattice planes were increased by the treatment, indicating a significant increasing in crystallinity after acidified sodium chlorite (3x) and sodium bicarbonate treatments. The peaks corresponding to (101) and (10$\underline{1}$) were not clearly observed in the control sample, probably due to interference from other components in the samples (e. g.

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lignin). After the treatment with acidified sodium chlorite (3x), these peaks could be significantly noticed, but not so obvious as those without extended treatment by sodium bicarbonate.

Based on FTIR analysis (Fig. 4), there are shown three representative chemical changes related to lignin removal, as assigned in Table 1.

Wavenumber (cm^{-1})	**Assignment**	**Reference(s)**
3450-3350	O-H stretching	Nelson and O'Connor, 1964
2901-2892	C-H stretching	Schwanninger et al., 2004
1745	Carbonyl bonds (associated with lignin side chain removal)	Kumar et al., 2009
1732	Alkyl esther from cell wall hemicellulose C=O; strong carbonyl groups in branched hemicellulose	Liu et al., 2005; Pandey, 1999; Sene et al., 1994
1650-1640	C=O vibration; Amide I, aromatics	Haberhauer et al., 1998
1638-1604	Doublet phenolics of remained lignin	Sene et al., 1994
1517-1516	Aromatic C-O stretching mode for lignin; guayacyl ring of lignin	Liu et al., 2005
1512	Aromatic C-O stretching mode for lignin; guayacyl ring of lignin; lignocellulose	Ouatmane et al., 2000
1430	CO_2 stretching; carboxylic acids	Smith et al.,1999
1375-1370	C-H stretch of cellulose	Liu et al., 2005; Stewart et al., 1995
1247-1242	C-O-H deformation and C-O stretching of phenolics	Stewart et al., 1995; Sene et al., 1994
1162-1159	Antisymmetric stretching C-O-C glycoside; C-O-C β-1,4 glycosil linkage of cellulose.	Liu et al., 2005; Michell, 1990
1109-1098	C-O vibration of crystalline cellulose; glucose ring stretch from cellulose	Pandey, 1999; Stewart et al., 1995
1060, 1035	C-O vibration of cellulose	Stewart et al., 1995
900-897	Amorphous cellulose vibration; glucose ring stretch	Pandey, 1999; Stewart et al., 1995

Table 1. Assignment of the main bands in FTIR spectra for rice straw

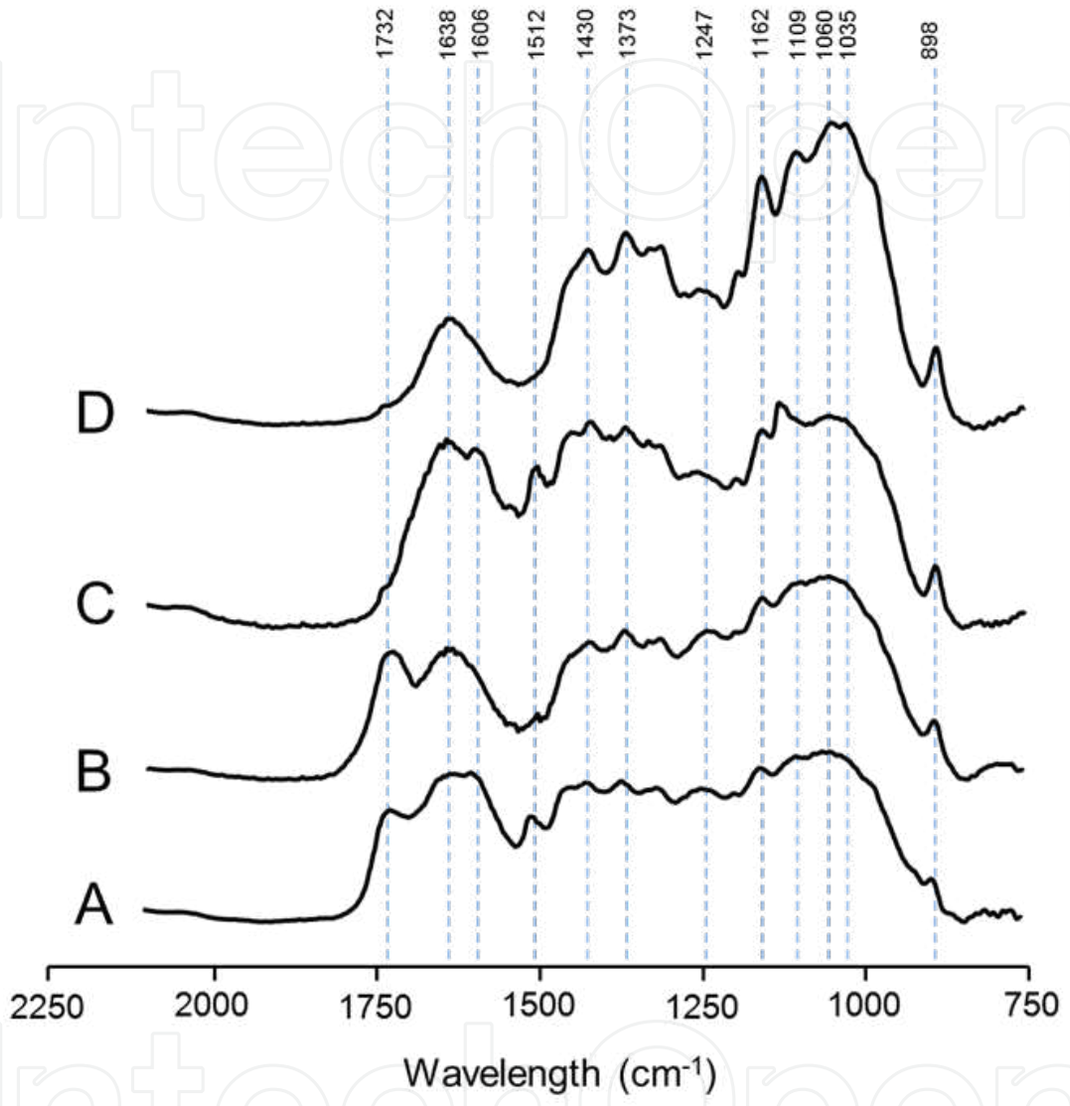

Figure 4. Chemical changes in rice straw solids determined by FTIR of wavelength ranged from 780 to 2200 (cm^{-1}). Symbols: (A) autoclaved rice straw, (B) rice straw after treated with acidified sodium chlorite (3x), (C) rice straw after treated with sodium bicarbonate, (D) rice straw after treated with acidified sodium chlorite (3x) and sodium bicarbonate.

1. The chemical changes in band position between 1745 and 1732 cm^{-1}. These are assigned to the carbonyl (C=O) stretching, attributed to aromatic skeletal vibrations in lignin structures (Kumar et al., 2009; Liu et al., 2005). Carbonyls mainly exist in the side chains of lignin structural units and are also an important functional group in the side chains. Particularly, the band position of 1732 cm^{-1} is attributed to the linkage of lignin side chain

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with branched hemicellulose. The significant change in these bands was almost unchanged by treatment with acidified sodium chlorite (3x) (B). After treated with sodium bicarbonate, these bands disappeared, indicating that the lignin linked to branched hemicellulose was removed from the cellulose structures (C, D), as also previously suggested (Liu and Wyman, 2004).

2. The chemical changes in band positions of 1606 cm^{-1} and 1638 cm^{-1}. The band at 1638 cm^{-1} is assigned to an aromatic stretch, and the band at 1606 cm^{-1} appears associated with the α-β double band of the propanoid side group in lignin-like structures (Sene et al., 1994). As shown in Fig. 4, The band at 1606 cm^{-1} was weak in delignified samples by acidified sodium chlorite treatment (3x) (B), and then disappeared after treated with sodium bicarbonate (C, D), indicating that lignin and its aggregates were also removed from the cellulose structure. Since the absorption band at 1638 cm^{-1} was often overlapped with the band assigned to absorbed water in cellulose (Chen et al., 1997; Gastaldi, et al., 1998), characteristic of the band at 1638 cm^{-1} may be similar to that at 1606 cm^{-1}.

3. The chemical changes in band position of 1512 cm^{-1}. This band is assigned to an aromatic C-O stretching mode in lignin (Ouatmane et al., 2000). The absorption band at 1512 cm^{-1} till remained in delignified samples by acidified sodium chlorite treatment (3x), and disappeared by sodium bicarbonate treatment. The chemical changes in band position between 1745 and 1732 cm^{-1} indicate the removal of integrated lignin, while the changes in band positions of 1606 and those of 1512 cm^{-1} indicate the removal of surface lignin. According to these results, it is clear that delignification by acidified sodium chlorite (3x) could efficiently remove surface lignin covered cellulose. However, to remove lignin completely, delignified rice straw must be treated with sodium bicarbonate.

3.2. Chlorine species produced during delignification process

Chlorine species produced in one time treating (1x) with acidified sodium chlorite for delignification were chlorite, chloride and chlorate ions and chlorine dioxide as shown in Fig. 5, in which rice straw and chemical lignin were prepared by equal weight and the control was treated without substrate. In general, sodium chlorite dissociates depending on pH in water and is converted to chlorite ion, in which it produces chlorine dioxide and chloride ion in acidic condition of pH 2 or less. In this study, the reaction was conducted in range of pH 4.5-4.8 under buffering by acetic acid and the main chemical species were chlorite with some chloride and little release of chlorine dioxide as observed in the control (Fig. 5.).

Chlorite is a strong oxidant and acts selectively on lignin (Ahlgren and Goring, 1971). In the delignification process of rice straw, lignin was clearly oxidized and removed by chlorite and then chlorite was reduced to chloride as confirmed by changes of chemical species in chemical lignin and rice straw (Fig. 5). Some chlorate was also produced. Meanwhile, about 20% of chlorite remained after one time treating in rice straw, even though 38% of lignin left as shown in (1x) of Fig. 1(A). This is because one time treating was not enough to remove the internal lignin of rice straw.

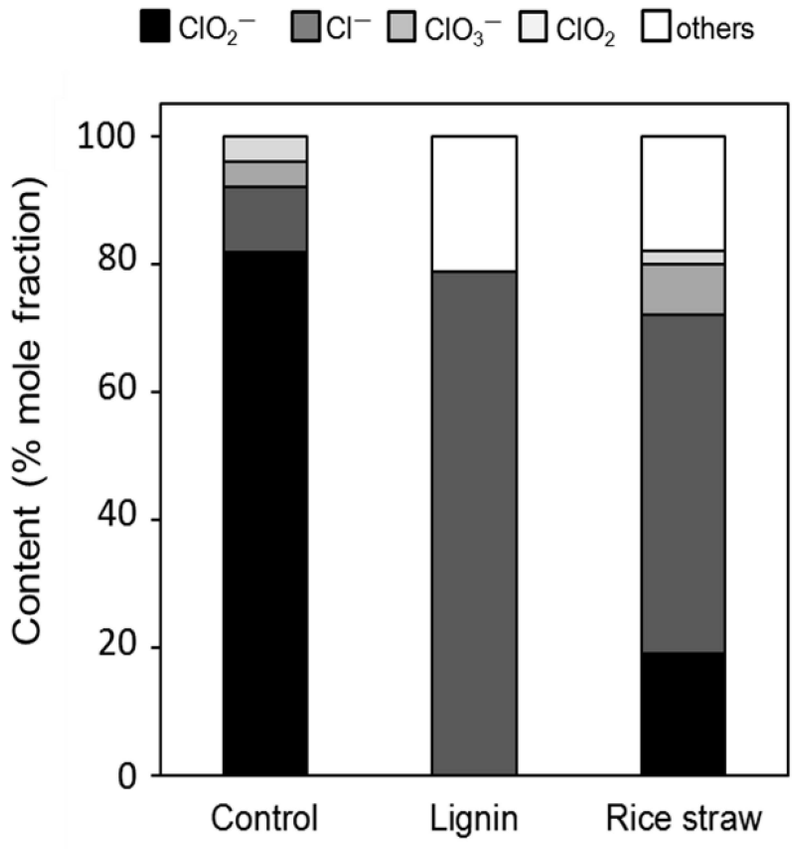

Figure 5. Formation of chlorite ion (ClO_2^-), chloride ion (Cl^-), chlorate ion (ClO_3^-) and chlorine dioxide (ClO_2) by oxidation of substrate using sodium chlorite as an oxidant. Rice straw and chemical lignin solids were used as substrates. Control means the reaction without any substrates.

3.3. Alteration in crystalline and chemical structures of microfibrils by pretreatment

Treatment with sodium bicarbonate also changes the properties of cellulose surface (Kwasniakova et al., 1996). As shown in Fig. 4, band position of 1430 cm^{-1} is assigned to CO_2 stretching for carboxylic groups on the surface of cellulose (Smith et al., 1999). The absorbance at this band position was significantly increased by treatment with sodium bicarbonate, indicating that the surface of cellulose microfibrils was carboxylated due to the treatment. The absorbance at 898 cm^{-1} is associated with the anti-symmetric out-of-phase ring stretch of amorphous cellulose (Stewart et al., 1995; Michell, 1990), and the 1060 and 1035 cm^{-1} bonds are related to C-O vibration of crystalline cellulose (Stewart et al., 1995). Both the crystalline (1035-1109 cm^{-1}) and amorphous (898 cm^{-1}) bands increased in intensity after treatment with sodium bicarbonate, suggesting that the sample had a higher percentage of crystalline cellulose, which we predicted that it was difficult to further hydrolyze with cellulase enzymes, particularly Cel7A. However, the saccharification yield of rice straw treated with sodium bicarbonate was significantly enhanced even though possessing high crystallinity. In this case, the carboxylation of cellulose surface supported the disintegration of cellulose structures, and allowed the enzyme to degrade the cellulose microfibrils.

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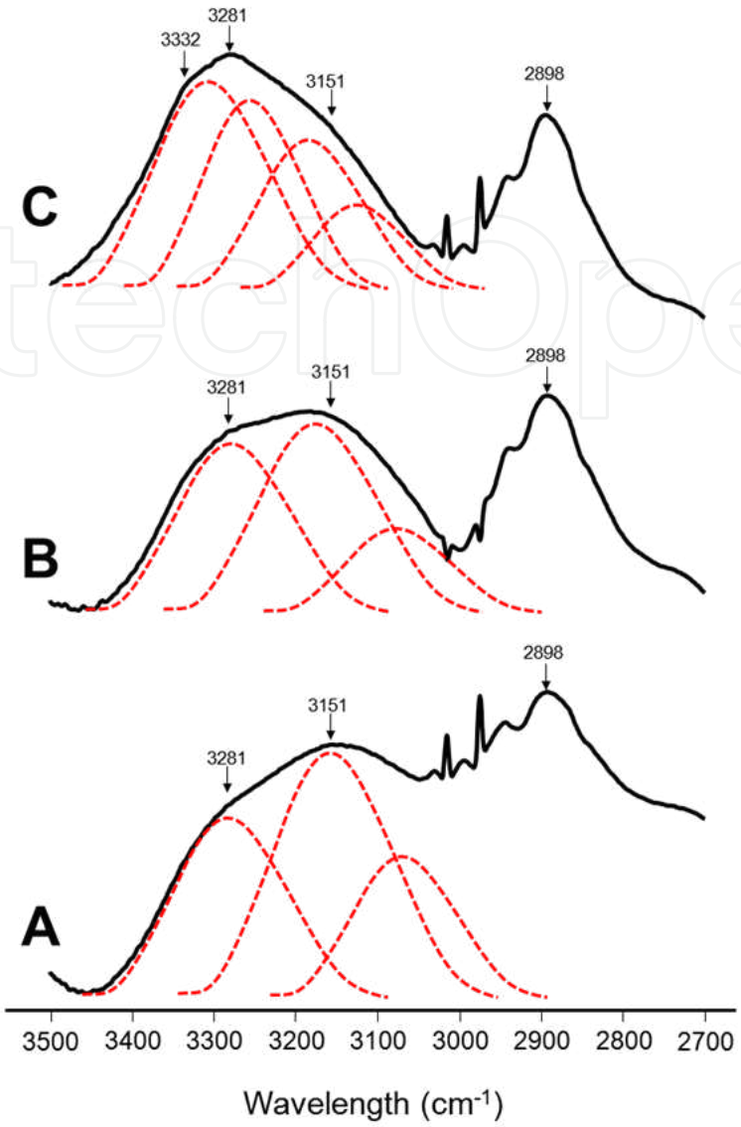

Figure 6. Chemical changes in rice straw solids determined by FTIR of wavelength ranged from 2700 to 3500 (cm^{-1}). Symbols: (A) autoclaved rice straw, (B) rice straw after treated with acidified sodium chlorite (3x), (C) rice straw after treated with acidified sodium chlorite (3x) and sodium bicarbonate.

Thermochemical treatments have a potential to change cellulose crystalline structure by disrupting inter/intra hydrogen bonding of cellulose chains (Zugenmaier, 2001). After treatment with sodium bicarbonate, the transformation of cellulose crystallinity from type I to type II was noticed, but it is not significant. As shown in Fig. 6, the typical changes in spectra length attributed to the transformation of crystal structure were, the decreasing of absorbance band at 3151 cm^{-1}, and the increasing of absorbance bands at 3281 and 3332 cm^{-1}, which were assigned to hydrogen-bonded OH stretching on the cellulose surface. Based on the previous study (Oh et al., 2005), there are three or four spectra overlapped in the weave length ranging from 2900 to 3400 cm^{-1} giving the characteristic of bands with corresponding with the transformation of crystal type, as indicated by red dashed line in Fig. 6. The increasing of absorbance

bands at 3281 and 3332 cm^{-1} was caused by the strong valence vibration of H bonded OH groups at O(2)H-O(6)H(intra) and O(3)H-O(5)H(intra), while the decreasing of absorbance band at 3151 cm^{-1} was caused by the weak valence vibration at O(6)H-O(3')H(inter) and O(6)H-O(2')H(inter), changing the stereochemistry at C2, C3, C5 and C6 inside the cellulose structure (Fig. 7). In case when the crystal type changed from type I to type II, the valence vibration at O(6)H-O(3')H(inter) and O(6)H-O(2')H(inter) became weak, whereas O(2)H-O(6)H(intra) and O(3)H-O(5)H(intra) became strong, due to the relocation of C-O changing the orientation of crystal. As shown in Fig. 6, the band at 3151 cm^{-1} was weak, but the bands at 3281 and 3332 cm^{-1} were strong, after treatment with sodium bicarbonate. By contrast, the treatment with acidified sodium chlorite (3x) only did not give the greatest changes at the length. Therefore, the treatment with acidified sodium chlorite (3x) could not change the crystal structure of rice straw cellulose to type II-like structure, unless combined with swelling by sodium bicarbonate.

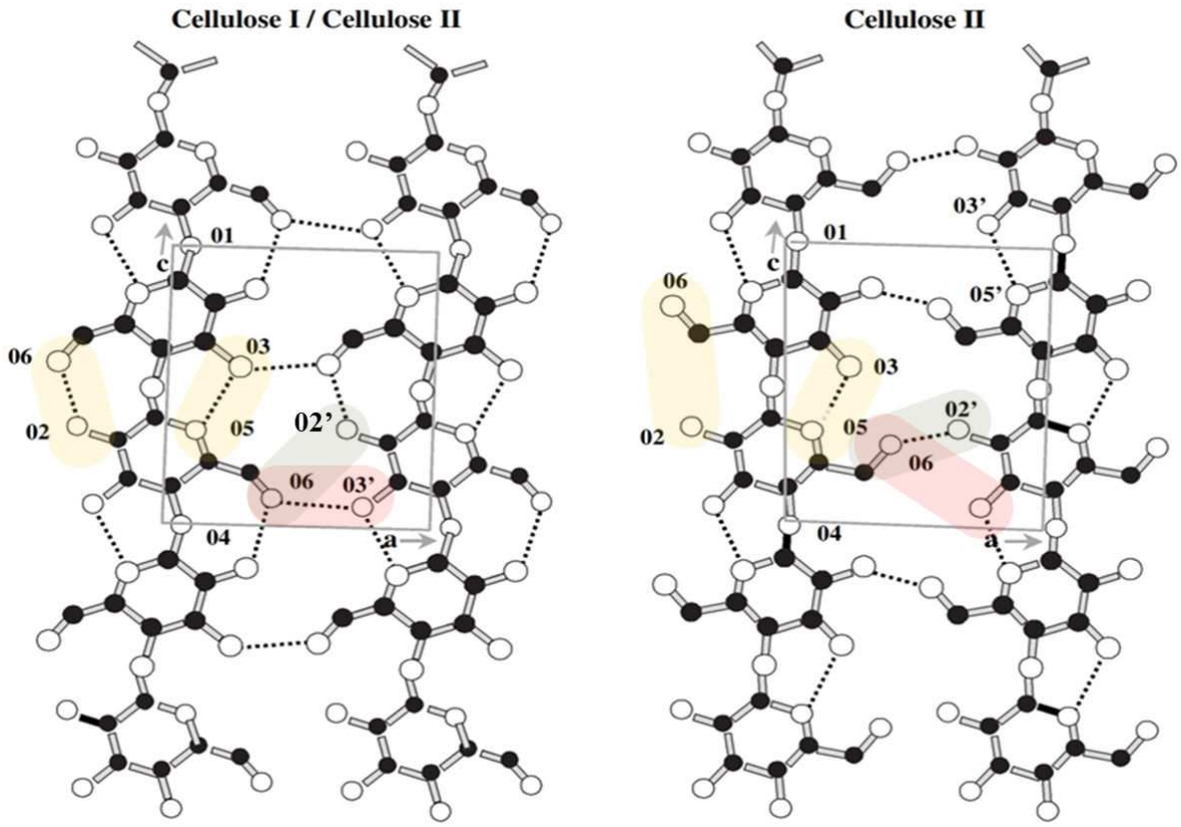

Figure 7. Proposed hydrogen-bonding patterns by Kolpak and Blackwell (1976): (a) 020 plane ('down' chains), (c) 020 plane ('up' chains).

3.4. Saccharification and fermentation of treated rice straw

Figure 8A shows the saccharification of rice straw, which was first delignified by acidified sodium chlorite (3x) only or alkaline treated by sodium bicarbonate only or treated with sodium bicarbonate after delignified by acidified sodium chlorite (3x). As a control, autoclaved rice straw with-

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out addition of any chemicals was used. The saccharification was performed under condition as mentioned in Materials and Methods section using commercially available enzymes mixture (Celluclast 1.5L and Novozym 188) at a low enzyme loading of 1/100 (g-enzymes/g-biomass solids). Single treatment with acidified sodium chlorite (3x) or with sodium bicarbonate could increase the saccharification rate of rice straw at 2.5 to 3-times higher than the control. However, high saccharification rate could be achieved from rice straw in case rice straw was treated with sodium bicarbonate after delignification by acidified sodium chlorite (3x). The rate was about 96% (wt.-reduced sugars/wt.-pretreated biomass), 4.2-times higher than the control, and even 1.5-times higher than the single treatments. This result indicates that the application of delignification and swelling processes on rice straw pretreatment can result in synergistic effect that enables broader success in achieving high enzymatic saccharification efficiency in processing rice straw to fermentable sugars, biofuels and value-added products.

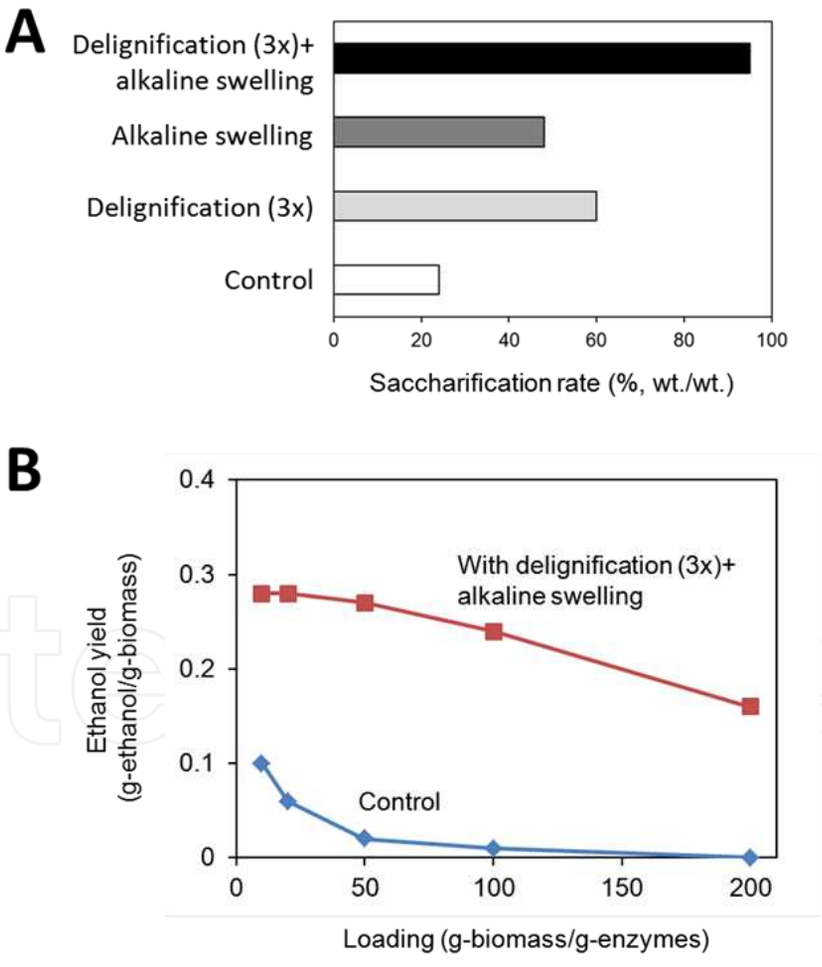

Figure 8. Saccharification (A) and fermentation (B) of treated rice straw. Saccharification was performed in 0.1M sodium acetate buffer (pH 4.8) for 3 days using commercial available cellulase enzyme mixtures (Novozym Celluclast 1.5L and 188) at enzyme loading of 1/100 (g-enzymes/g-biomass). Fermentation was performed anaerobically at 30 ºC until no longer ethanol produced. Untreated rice straw was used as a substrate in all control experiments.

To test the feasibility of using the treated rice straw as a substrate in simultaneous saccharification and fermentation (SSF), the fermentations were performed at solid loadings of 50, 100, 150 and 200 (g-biomass/g-enzymes) under condition as mentioned in Materials and Methods section. The fermentations of untreated rice straw at similar solid loadings were used as controls. Figure 8B shows the yield of ethanol highly obtained from fermentation of treated (solid line) and untreated (dashed line) rice straws by using *S. cerevisiae* NBRC2114 as a strain. As clearly form the figure, high ethanol yield of 0.28 (g-ethanol/g-biomass) was obtained in case of treated rice straw. Since the fermentation of untreated rice straw could only produce ethanol at 0.12 (g-ethanol/g-biomass), the treatment with sodium bicarbonate after delignification by acidified sodium chlorite (3x) remarkably enhanced efficiency of fermentation by 2.3-times (in this study), as indicated by the ethanol yield. In spite of the fact that the ethanol yields gradually decreased as solid loading increased, SSF of treated rice straw was still enabled, as indicated by the ethanol yield of 0.16 (g-ethanol/g-biomass) achieved at solid loading of 200 (g-biomass/g-enzymes). By contrast, it was difficult to perform SSF of untreated rice straw even at solid loading of 50 (g-biomass/g-enzymes). According to these results, we conclude that the treatment with sodium bicarbonate after delignification by acidified sodium chlorite (3x) is effective in enhancing the enzymatic saccharification of rice straw in SSF process, particularly at high biomass solid with low enzyme loadings.

3.5. Inside the chemical reactions involved in the pretreatment process

Figure 9 shows a proposed conceptual model for the mechanism of enhanced enzymatic saccharification of rice straw by delignification with acidified sodium chlorite and by swelling and surface rearrangement with sodium bicarbonate treatment, as described in this study. During the delignification process by acidified sodium chlorite, the hydroxyl groups and reducing end groups of cellulose can also be oxidized (Fengel and Wegener, 1984).

Chemical oxidation of the reducing-ends of cellulose could negatively interact with Cel7A, a reducing-end targeting cellobiohydrolase (Barr et al., 1996). In fact, the treatment by acidified sodium chlorite could successfully improve the efficiency of saccharification on treated rice straw three-times compared with the control (Fig. 8A). As reported by Ishizawa et al. (2009), the acidified sodium chlorite treatment has no detectable effect on treated rice straw three-times compared with the control (Fig. 8A). As reported by Ishizawa et al. (2009), the acidified sodium chlorite treatment has no detectable effect on digestibility or accessibility of crystalline cellulose by Cel7A. However, amorphous cellulose was more susceptible to the oxidative treatment and showed a slight decreasing in the initial cellulose conversion and enzyme binding levels using Cel7A.

In this study, the digestion of delignified rice straw by cellulase enzyme mixture (more than 60% of total protein was Cel7A, according to Nummi et al., 1983) was remarkably enhanced, probably not only due to the removal of surface lignin, but also due to the protection of other amorphous parts of cellulose by its rigid structure. It could be proven by XRD analysis as shown in Fig. 3, the slightly increasing in its crystallinity after treated by acidified sodium chlorite (3x), indicating that most amorphous cellulose was placed inside the structure untouched by chlorite even after the treatment.

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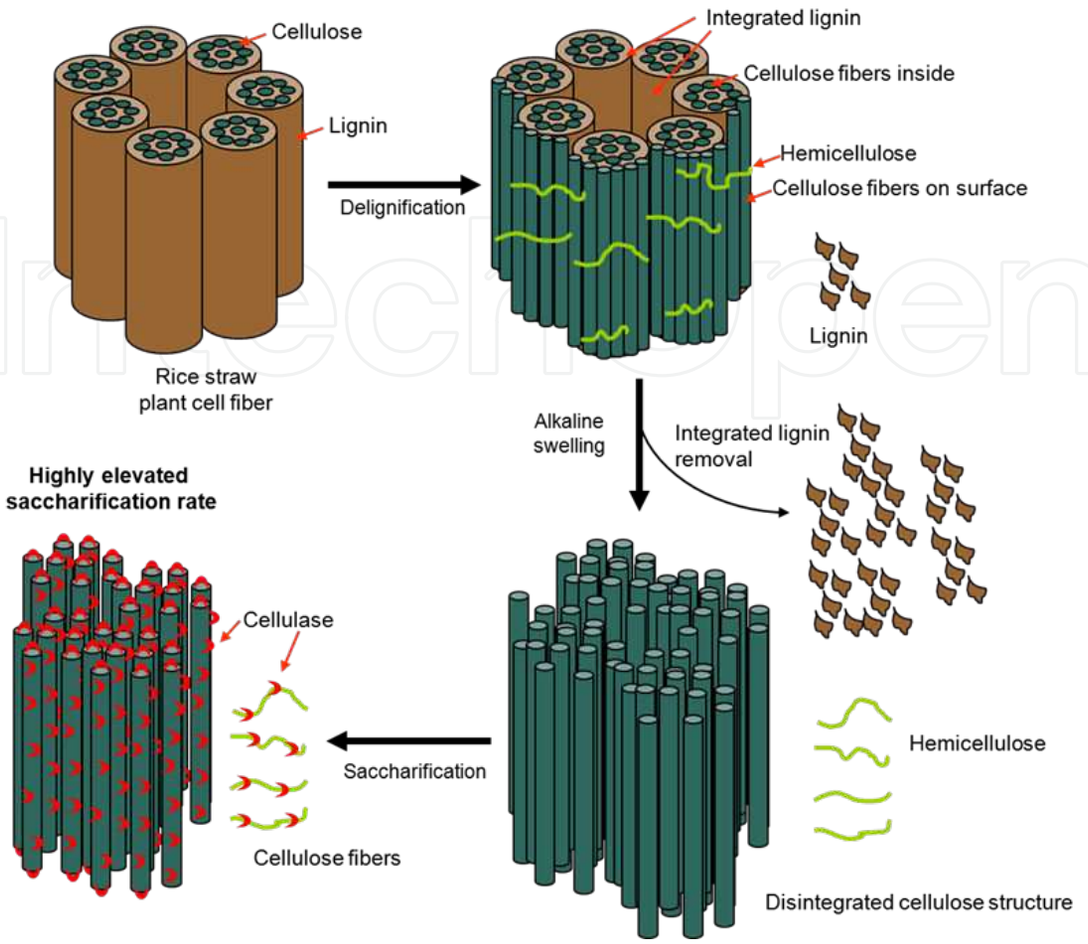

Figure 9. A proposed conceptual model for the mechanism of enhanced enzymatic saccharification of rice straw by delignification with acidified sodium chlorite and by swelling and surface rearrangement with sodium bicarbonate treatment.

Furthermore, the alkaline treatment (swelling) by sodium bicarbonate has a potential to dramatically enhance the saccharification of delignified rice straw, as shown in Fig. 9. Generally, the alkaline treatment resulted in remarkable decreasing of hemicellulose due to lignin removal (Liu and Wyman, 2004). The recovery of residual rice straw after sodium hydroxide treatment was low compared with sodium bicarbonate, even though alkaline treatment by sodium hydroxide is widely used to obtain fiber swelling upon mercerization. Taniguchi et al. (1982) reported that the saccharification of sodium hydroxide-treated rice straw was low compared to that treated with sodium bicarbonate. One possible explanation for this phenomenon is that the near complete removal of lignin allows adjacent hemicellulose-free cellulose microfibrils to aggregate upon elimination of the lignin spacer (Duchesne et al., 2001; Oksanen et al., 1997). Similar results have been reported in the literature (Fan et al., 1980), showing that the hydrolysis rate of wheat straw increases with delignification up to about 50%, after which cellulose hydrolysis increases only slightly. By contrast, the alkaline treatment by sodium bicarbonate allows the swelling similar to that with sodium hydroxide, but the difference is on the chemical modification of cellulose fiber surface by carboxylation

with bicarbonate treatment (Kwasniakova et al., 1996). As a conclusion, the carboxylation on the cellulose surface could prevent the aggregation of cellulose fibrils.

4. Conclusion

The synergistic effect of delignification and swelling on the enzymatic saccharification of rice straw was evaluated by residual lignin estimation, x-ray diffraction, FTIR spectrophotometry, SEM as indirect methods, by enzymatic saccharification using purified or commercial available cellulase enzymes as a direct method. The removal of total lignin, including surface and internal lignin, is an indispensable pretreatment for achieving high efficient enzymatic saccharification of rice straw. Treatment with acidified sodium chlorite (3x) could remove surface lignin only, but internal lignin remained. By combination with the swelling using sodium bicarbonate, total lignin could be removed. Swelling with sodium bicarbonate did not result in the removal of total lignin only, yet it could breakdown the cellulose structure to release disintegrated cellulose fibrils and indeed change the chemical properties of cellulose surface by decarboxylation. By treated rice straw with acidified sodium chlorite (3x) and then with sodium bicarbonate, the saccharification yield was remarkable increased compared with that only by acidified sodium chlorite (3x) only. This indicates that delignification could not enhance the saccharification without the synergistic effect of the combination with swelling by alkaline treatment.

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